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13. ABSTRACT *(Maximum 200*

Progress has been made at the pre-clinical level in the study of iron deprivation as a potential treatment modality for metastatic breast cancer. The MDA-MB-231 cell line is sensitive to combined iron deprivation treatment in vitro but does not appear to be sensitive in vivo. The SK-BR-3 cell is also sensitive in vitro but could not be evaluated in vivo. The contrasting fact that iron deprivation induces apoptosis in the 38C13 lymphoma (and inhibits growth in vitro and in vivo) is now being pursued with the hypothesis that a response to iron deprivation requires an intact p53 gene. The exon/intron structure of the human transferrin gene has been determined and this will facilitate experiments to knock out the gene in cell lines and thus allow the determination of whether it is necessary for tumor growth in vivo. These genetic studies have incidentally created new opportunities to study the possible functional roles of polymorphisms in the transferrin receptor gene and the evolutionary relationship between the transferrin receptor gene and other genes.

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John D Kemp 2/21/99

PI - Signature Date

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Introduction

The problem that has been addressed by this research is the need for improved therapy of metastatic breast cancer. There have been, and continue to be, clear indications that iron deprivation treatment is useful clinically against several tumor types. Although we were able to show that breast cancer cell lines were sensitive to iron deprivation treatments in vitro, we were unable to demonstrate sensitivity of established tumors derived from the MDA-MB-231 cell line in vivo with a treatment protocol that was demonstrably effective against murine and human lymphomas in vivo. While this result is disappointing, it does not necessarily mean that all iron deprivation treatment protocols will be ineffective against all metastatic breast cancers.

Body

In relation to the pursuit of Task #1 in our Statement of Work, we were able to quantify the sensitivity of two breast cancer cell lines (SKBR-3 and MDA-MB-231) to combined treatment with deferoxamine (DFO) and a pair of IgG monoclonal antibodies against the human transferrin receptor. This work has been published (Pathobiology 63:65-70, 1998). This then led, as we had hoped it would, to the performance of in vivo experiments as detailed under Task #3 below. Although we initially had some difficulty in getting the MCF-7 cell line stabilized for testing in vitro, we eventually were able to proceed with it. We then elected to focus on MCF-7 in relation to the pursuit of Task #5 as will be described below.

In relation to Task #2 we noted in our report for 1995 that we had established that the IgG monoclonal antibody pair does cause receptor down regulation with the SKBR-3 and MDA-MB-231 cell lines and this was published in the aforementioned paper in Pathobiology (63:65-70, 1995). Of interest was the fact that the cell line which showed the greatest degree of receptor down-regulation also showed the greatest degree of growth inhibition in vitro. This fact is potentially relevant to the interpretation of the experiments described for Task #3 below.

As was noted in our report for 1996, an unanticipated benefit of our work came in the form of a collaboration with Dr. Jan Kovar, who showed that iron deprivation specifically induced apoptosis in the murine lymphoma 38C13 (Pathobiology 65:61, 1997). This work is now being followed up in a collaboration with Dr. Kovar by testing the hypothesis that a normally functioning p53 gene must be present in order for iron deprivation to have maximal effect. This hypothesis flows from prior observations that hypoxia induces p53 and that the effects of iron deprivation are in many ways similar to those of hypoxia. This work clearly has implications for future work with iron deprivation and breast cancer and we are currently planning new grant applications to pursue those implications.

We noted in our report for 1996 that other groups had continued to produce evidence that the effects of deferoxamine could be stoichiometrically reversed by iron in various experimental systems. Because of this, the question seemed essentially resolved, and we elected not to pursue it and to focus our energies on other tasks instead.

In relation to Task #3, we tested both SKBR-3 and MDA-MB-231 cell lines for their in vivo growth behavior. MDA-MB-231 grew most rapidly and became our first target for in vivo treatments. As noted in our 1996 report we had some difficulty in acquiring enough of the monoclonal antibodies for treatment but, after consultation with Drs. Ray Taetle and Toby Hecht, we succeeded. As noted in our report for 1997 we did test the MDA-MB-231 cell line for inhibition of growth and saw no meaningful effect. This was confirmed in a second experiment. These data were presented at the Era of Hope Meeting in Washington. We had hoped to extend those experiments with the SKBR-3 cell line since, as noted above, it showed more sensitivity to iron deprivation in vitro. However, this tumor never produced a stable and predictable growth pattern in vivo during 1997 or 1998 and was therefore not useful for that purpose. The question still remains, therefore, as to whether cell lines that are more sensitive to iron deprivation in vitro might exhibit measurable sensitivity to iron deprivation in vivo.

In relation to Task #4, our research has provided some novel and unexpected insights which have created new questions about iron deprivation and gene regulation. As was noted in our report for 1996, combined treatment produced elevated ceruloplasmin levels in all mice. This acute phase reactant has been linked to IL-6 and, as was noted in our report for 1997, treated mice did exhibit elevated IL-6 levels. A third of the mice also exhibited elevated nitric oxide levels. The latter observation appears to make sense in the context of prior observations which showed that iron deprivation could induce the nitric oxide synthase gene. We therefore believe that we now have better insights into the toxicity associated with combined DFO/monoclonal antibody iron deprivation treatment and that new grant applications can be written to pursue these insights.

Also of significant interest are the effects of monoclonal antibodies alone and we reported on these in our 1997 report. Anti-transferrin receptor monoclonal antibodies are capable of virtually complete inhibition of the generation of cytotoxic T lymphocytes in vivo and can cause some degree of inhibition of skin graft rejection. This finding could, in principle, be of therapeutic interest in breast cancer patients who have undergone bone marrow transplantation and are experiencing acute graft versus host disease. We are now considering the possibility of new grant applications to follow up on these observations.

In relation to Task #5, a set of studies was undertaken with the tamoxifen sensitive MCF-7 cell line as described in our report for 1996. Those studies showed that the DFO/ tamoxifen interaction was subadditive or antagonistic. It was proposed that the reduction in the stimulus for entry into the cell cycle caused by tamoxifen exposure might have impeded the effects of iron deprivation which, at limiting doses, are most likely to occur in S phase.

As we realized that we had made substantial progress towards all of our original tasks, that new approaches were called for, and that future progress was likely to involve molecular biology, we elected to develop more expertise in this area. We therefore devoted some of our energy to a project to analyze the exon-intron

structure of the human transferrin receptor gene (published in Gene 199:123-131, 1997). This work has now paved the way for the construction of targeting vectors to knock out the transferrin receptor gene in breast cancer cell lines. The purpose of this will be to test the hypothesis that the transferrin receptor is necessary for in vivo growth of breast cancer cells. This work incidentally led to the identification of a protein sequence polymorphism in exon 4 of the human transferrin receptor (described in the Gene paper). This has created the possibility of asking whether this polymorphism is correlated with normal variation in standard indices of iron economy, including the level of circulating soluble transferrin receptor. To pursue this, we have now begun to collaborate with Drs. Becky Biga and Trudy Burns (in Preventive Medicine at the University of Iowa) to determine the allele status of a cohort of individuals in the Muscatine Heart Project whose basic iron indices are known. The gene sequence data is also now being employed in a very recently established collaboration with a group in Switzerland in an attempt to detect other human polymorphisms. The sequencing data has also suggested that the homology between the transferrin receptor gene and the gene for the prostate specific membrane antigen is much greater than previously realized. We are now planning to test the hypothesis that the two genes are part of a family that arose out of an ancient gene duplication event.

Conclusions

1. Iron deprivation treatments do inhibit breast cancer cell lines in vitro.
2. Anti-transferrin receptor monoclonal antibodies do cause receptor down-modulation in breast cancer cell lines and the degree of such down-modulation may predict the relative in vitro sensitivity of a given cell line.
3. A breast cancer cell line that was sensitive to iron deprivation in vitro was not obviously sensitive to iron deprivation in treatment in vivo, but the question remains as to whether all breast cancer cell lines will behave in this manner. If the latter turns out to be the case, then growth in vivo may be associated with greater tumor access to treatment resistant iron stores than is the case in vitro, greater cellular resistance to the treatment protocol itself, or a combination of the two.

4. Iron deprivation appears, somewhat unexpectedly, to be able to induce the expression of the acute phase reactant ceruloplasmin (possibly as a result of increased IL-6 production) as well as increase the production of nitric oxide. Once these early observations are studied in more depth, it may be possible to develop a more detailed model to account for the toxicity of iron deprivation treatment we previously observed in animal model studies (Cancer Research 55:3817-3824, 1995). In addition, monoclonal antibodies against the transferrin receptor inhibit the generation of cytotoxic T lymphocytes and can, in some cases, retard skin graft rejection. Such findings might, in principle, be relevant to the treatment of acute graft versus host disease in breast cancer patients undergoing bone marrow transplantation.

5. DFO and tamoxifen appear to interact in a subadditive for antagonistic fashion, possibly resulting from a reduced drive for entry into the cell cycle acting to restrict the opportunity for an S phase inhibitory agent to work.

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